

# Anticarcinogenic effect of phytic acid (IP<sub>6</sub>): Apoptosis as a possible mechanism of action<sup>☆</sup>

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## Abstract

Several in vivo and in vitro studies provide convincing evidence for the anticarcinogenic properties of phytic acid (PA) (Inositol hexaphosphate, IP<sub>6</sub>). The objectives of this investigation were to elucidate the effects of PA on suppression of colonic aberrant crypt foci (ACF) and to study the inhibitory effect of IP<sub>6</sub> on human colon carcinoma, CaCo-2 cell line. Fisher 344 male, weanling rats were divided into 3 groups of 15 each and were fed control diet (AIN 93 G-C) and C + (1 or 2 g/100 g PA in water) for 13 weeks. Rats received 2 s.c. injections of azoxymethane (AOM) in saline at 16 mg/kg body weight at 7 and 8 weeks of age. There was a 36% and 42% reduction in ACF in 1 and 2 g/100 g PA groups compared to control group ( $P < 0.001$ ). Cytotoxic effect of IP<sub>6</sub> was evaluated on CaCo-2 cell line at concentrations of 0.25–4 g mol/l using lactate dehydrogenase (LDH) assay (LDH released from the cytosol of damaged cells into the supernatant), histone-associated DNA fragmentation assay using a cell death detection ELISA<sup>®</sup> kit and microscopic analysis. The cells were treated with 0.25, 0.5, 1.0, 2.0, 4 g mol/l of IP<sub>6</sub> and incubated for 24 and 48 h. After 24 h of incubation, LDH release ranged from 13.55% to 44.70%. Histone-associated DNA fragmentation (an assay based on the quantitative sandwich-enzyme-immunoassay-principle directed against DNA and histones which allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates) was also dose dependent. CaCo-2 cells grown on slide flaskets in the presence of 0.25 g mol/l IP<sub>6</sub> showed membrane blebbing (zeiosis) characteristic of apoptotic activity. Results of this study indicate that IP<sub>6</sub> might exert anticarcinogenic activity through induction of apoptosis.

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## 1. Introduction

Cancer of the colon is one of the leading causes of cancer morbidity and mortality among men and women in the Western countries, including the United States (Parker, Tong, Bolden, & Wingo, 1997). Epidemiologic studies suggest that increased consumption of fruits and vegetables and high total dietary fiber reduce the risk of development

of colon cancer (Howe, Benito, & Castello, 1992; Steinmetz & Porter, 1991; Potter, 1993). Human metabolic and laboratory animal model studies indicate that beneficial effects of dietary fiber in relation to colon cancer development depend on the physical properties of the fiber and amount consumed (Reddy, Engle, Simi, & Goldman, 1992; Reddy, 1995).

Phytic acid (PA) (IP<sub>6</sub> or Ins P<sub>6</sub>) is present in foods at concentrations of 0.1–6.0 g/100 g (Berridge, 1987; Reddy, Sathe, & Salunkhe, 1982). Phytates are found as crystalline globoids inside protein bodies in the cotyledon of legumes and oilseeds or in the bran of cereal grains (Reddy et al., 1982; Challa, Rao, & Reddy, 1997). Inositol hexaphosphate is a naturally occurring phosphorylated carbohydrate, found

*Abbreviations:* ACF, Aberrant crypt foci; AOM, Azoxymethane; IP<sub>6</sub>, Inositol hexaphosphate

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in plants and in almost all mammalian cells (Graf & Eaton, 1993).

PA consists of a myo-inositol ring with six phosphate moieties attached (Graf & Eaton, 1993). It serves as the major storage form of phosphorus in the seed, as well as being a natural antioxidant by its chelating properties and reducing the catalytic activities of many divalent transition metals (Thompson & Zhang, 1991; Thompson, 1993). It is the chelating ability of PA that has been suggested to suppress iron-mediated oxidation in the colon, thereby reducing colon cancer risk (Graf & Eaton, 1993; Graf, Empson, & Eaton, 1987; Nelson, 1992; Shamsuddin, 1992). PA may also bind proteins and starches present in the diet and so affect their function, solubility, digestibility and absorption (Richard & Thompson, 1997), which in turn may affect the colonic environment, for example, by stimulating short chain fatty acid (SCFA) production from fermentation of the trapped carbohydrates. The dephosphorylation and rephosphorylation of IP<sub>6</sub> and Inositol are important events, particularly in signal transduction (Graf & Eaton, 1993).

Aberrant crypt foci (ACF), which are recognized as early preneoplastic lesions in the colon, have consistently been observed in experimentally induced colon carcinogenesis in laboratory animals and in the colonic mucosa of patients with colon cancer (McLellan, Medline, & Bird, 1991; Pretlow, Oiriodian, Somich, & Pretlow, 1992). Aberrant crypts are putative precursor lesions from which adenomas and carcinomas may develop in the colon. Several inhibitors of ACF formation have been shown to reduce the incidence of colon tumors in laboratory animals (Wargovich et al., 1996), suggesting that ACF induction can be used to evaluate novel agents for their potential chemopreventive properties against colon cancer.

Anticancer action of IP<sub>6</sub> has been demonstrated both in vivo and in vitro which is based on the hypothesis that exogenously administered IP<sub>6</sub> may be internalized, dephosphorylated to IP<sub>1–5</sub> and inhibit cell growth (Shamsuddin, 1992). IP<sub>6</sub> appears to be affective at both pre- and post- initiation stages of carcinogenesis. IP<sub>6</sub> can lower the cell proliferative activity and tumor incidence. In vivo IP<sub>6</sub> inhibits human cancer cell growth with induction of cell differentiation (Shamsuddin, Vucenick, & Cole, 1997; Vucenick, Kalebic, Tantivejkul, & Shamsuddin, 1998).

A novel anticancer function of IP<sub>6</sub> has recently been shown by others (Shamsuddin et al., 1997; Shamsuddin, 1995; Shamsuddin, Elsayed, & Ullah, 1988) which supports our hypothesis that IP<sub>6</sub> exerts its antineoplastic activity by entering into cellular Inositol phosphate pool, and affecting common cellular signal transduction pathways and therefore decreases cell proliferation (Graf & Eaton, 1993).

The objectives of this study were to elucidate the effects of IP<sub>6</sub> on suppression of colonic aberrant crypt foci (ACF) and to study the inhibitory effects of IP<sub>6</sub> on human colon cancer cell line CaCo-2.

## 2. Materials and methods

### 2.1. Animal experiment

#### 2.1.1. Animals, housing and diets

Forty-five male Fisher 344 weanling rats were obtained from Charles River Breeding Inc. (Wilmington, MA, USA) and housed in stainless-steel cages at 2 rats/cage. After 1-week period of acclimatization 3 groups of 15 rats each were assigned to 3 dietary treatments (0, 1, 2 g/100 g PA in water and fed AIN 93G diet (Table 1) for 13 weeks. PA was purchased from Sigma Chemical Company (St. Louis, MO, USA) and prepared fresh daily at 1 and 2 g/100 ml concentrations in deionized water. These levels were selected based on previous research conducted in our laboratory (Challa et al., 1997) and other published research (Shamsuddin et al., 1988). The PA solution was heated to allow it to dissolve completely, and cooled to room temperature and the pH was adjusted to 7.0. All diets were mixed on a weekly basis and stored at 4 °C until used. Dietary ingredients were obtained from ICN (Costa Mesa, CA, USA). Temperature and relative humidity were maintained at 21 °C ± 1 °C and 50%, respectively. Light and dark cycles were at 12 h each. Feed and water were provided ad libitum. Weekly body weights and daily feed intakes were recorded. The diets were prepared fresh every week and stored at 4 °C until fed. All the protocols involving rats were approved by the Institutional Animal Care and Use Committee of Alabama A&M University, Normal, AL, USA.

#### 2.1.2. Carcinogen injection

All animals received a subcutaneous injection of azoxymethane (AOM) in saline (Sigma, St. Louis, MO, USA) at 16 mg/kg body weight at 7 and 8 weeks of age according to the standard protocol. Additional 5 rats received saline injection only. Animals were killed using CO<sub>2</sub> euthanasia at 17 weeks of age.

#### 2.1.3. Colon sample collection

Colons of rats from both groups were removed and flushed with potassium phosphate buffer (0.1 mol/l, pH 7.2)

Table 1  
Composition of the diets

Ingredients (g/kg)	Control
Corn starch	397.5
Casein (85 g/100 g protein)	200
Dextrin	132
Sucrose	100
Fiber <sup>a</sup>	50
Oil (soybean, no additives)	70
Mineral mix (AIN 93G MX) <sup>b</sup>	35
Vitamin mix (AIN-93 VX) <sup>b</sup>	10
Cystine	3
Choline bitartrate (41.1 g/100 g choline)	2.5

<sup>a</sup>Solk-FlocR, 200 FCC (FS&D, St. Louis, MO, USA) or equivalent.

<sup>b</sup>Formulation of diets based on AIN 93G (Reeves et al., 1993).

and were analysed for ACF which are preneoplastic lesions and are excellent intermediate markers of colon tumorigenesis.

#### 2.1.4. Counting the ACF

Colons were split open longitudinally and placed on a filter paper with their luminal surface open and exposed. Another filter paper was placed on top of the luminal surface and fixed overnight using 10 g/100 g buffered formalin. Each fixed colon was cut into proximal and distal portions of equal length and each portion was further cut into 2 cm long segments. Each segment was placed in a Petri dish and stained using 0.5 g/100 g methylene blue solution for 5 min. The segments were transferred to another Petri dish containing buffer to wash excess stain and then examined under a light microscope to score the total number of ACF as well as the number of crypts per focus according to the procedure described by Bird and Good (2000). Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, significantly increased distance from lamina to basal surface of cells and the easily discernible pericryptal zone. Colons of rats from each dietary group were prepared for counting ACF as described by Bird (1987).

#### 2.1.5. Statistical analysis

Body weights and ACF were compared between the animals. Data were analysed using the SAS (1997) Statistical program; means were separated using the Tukey's Studentized Range Test Differences were considered statistically significant at  $P < 0.05$ .

#### 2.1.6. Cell culture Study

Inositol hexaphosphate (Dodecasodium salt, sodium phytate [ $\text{IP}_6$ ]) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solution (200 g mol/l, pH 7.0) was prepared fresh each time before use in sterilized deionized distilled water, the pH adjusted with HCl to 7.0 and sterilized by passing through a 0.22  $\mu\text{m}$  membrane filter. Dilutions of 0.25–40.0 g mol/l in saline were prepared from the stock solution. The saline served as negative control. CaCo-2 human colon carcinoma cells were obtained from the American Type Tissue Culture Co. MD(ATCC). Cells were maintained in Dulbecco's Modified Eagle's Media (DMEM) with 10 g/100 g fetal bovine serum. For assay,  $5 \times 10^4$  cells/well/100  $\mu\text{l}$  were seeded to a

24 well culture plate and incubated at 37 °C, 70%  $\text{CO}_2$ , till development of a monolayer. After the development of a monolayer 400  $\mu\text{l}$  of fresh medium (DMEM- serum free) was added to the wells and different concentrations of  $\text{IP}_6$  (0.25–40.0 g mol/l) made upto 100  $\mu\text{l}$  with saline were added and incubated for 24 and 48 h. The medium alone served as a negative control and saline served as a positive control. This experiment was carried out in replicates of 2 wells/concentration. After 24 h lactate dehydrogenase (LDH) release using a Cytotoxicity Detection Kit (obtained from Boehringer Mannheim, Indianapolis, IN, USA) was carried out. It is a colorimetric assay for the quantification of cell death and lysis, based on the measurement of LDH released from the cytosol of damaged cells into the supernatant. Histone-associated DNA fragmentation assay using a cell death detection ELISA<sup>®</sup> kit was carried out. The assay is based on the quantitative sandwich-enzyme-immunoassay-principle directed against DNA and histones. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Spectrophotometric analysis was carried out using an ELISA plate reader at 540 nm for the LDH assay and at 405 nm for the histone-related DNA fragmentation assay.

#### 2.1.7. Microscopic analysis

CaCo-2 cells were grown on slide flaskets in the presence of 0.25–4.0 g mol/l  $\text{IP}_6$  and incubated for 24 and 48 h and stained using Geimsa stain to determine any apoptotic activity.

### 3. Results and discussion

#### 3.1. Animal model

The present study was conducted to evaluate the potential inhibitory properties of PA ( $\text{IP}_6$ ) on ACF formation in the colon.

##### 3.1.1. Feed intake and body weight

There were no significant differences in mean feed intake for all the 3 groups (Table 2). Body weights for rats fed 2 g/100 g PA in water was significantly lower than the control although the group fed 1 g/100 g PA did not show any difference compared to controls (Table 2).

Table 2  
Effect of phytic acid on body weight, feed intake and fluid intake in Fisher 344 rats<sup>a</sup>

Treatment	N	Body weight gain (g/13 weeks)	Feed intake (g/rat/days)	Fluid intake (ml/rat/days)
Control	15	278.9 $\pm$ 5.8	17.5 $\pm$ 1.1	17.9 $\pm$ 0.6
1 g/100 g PA	15	269.8 $\pm$ 4.5	16.8 $\pm$ 0.9	17.8 $\pm$ 0.9
2 g/100 g PA	15	242.5 $\pm$ 4.9*	15.9 $\pm$ 1.0*	17.2 $\pm$ 1.0

<sup>a</sup>Values are Means  $\pm$  SEM.

\*Different ( $P < 0.05$ ) by Tukey's studentized test.

### 3.1.2. Aberrant crypt foci (ACF)

The animals administered saline (vehicle), showed no evidence of ACF formation in the colon. In the rats fed the control diet (AIN 93 G), AOM induced an average of 153 ACF/colon. The analysis showed that PA significantly reduced the number of ACF in the colon ( $P < 0.001$ ) when fed in water at the 1 and 2 g/100 g levels. This data is consistent with the results reported by Challa et al. (1997). There is a significant difference ( $P < 0.001$ ) between ACF levels in the distal and proximal colon. These data are consistent with the reports that the distal colon shows a greater incidence of colorectal cancer than the proximal colon in humans. The incidence of AOM-induced ACF was 61% in the 1 g/100 g IP<sub>6</sub> fed rats and 57.9% in the 2 g/100 g IP<sub>6</sub> group (Fig. 1). The greatest reduction (42.1%) in AOM-induced ACF was seen in the rats fed 2 g/100 g IP<sub>6</sub> in water. There was a 39% reduction in the distal colon and 51.9% in the proximal colon. A lower but similar reduction (32.6%) was seen in the 1 g/100 g IP<sub>6</sub> fed rats (Fig. 2). A reduction of 35.5% and 38% in total crypts was seen in the rats fed 1 and 2 g/100 g IP<sub>6</sub>, respectively (Table 3). PA may be acting at the signal transduction and cell proliferative stages (promotional stages) in reducing tumorigenesis (Shamsuddin, 1995). Pretlow et al. (1992) also reported a reduction in the number of ACF in the rat colon by IP<sub>6</sub>, administered in drinking water. They also reported a higher number of ACF containing 4 crypts/focus which was also seen in our study.

Compared to the rats fed PA, rats who were fed 10 g/100 g inulin (Verghese, Rao, Chawan, Williams, & Shackelford, 2002) had reductions in colonic ACF 62.5% in the proximal and by 60.1% in the distal colon with an overall 60.1% reduction in ACF ( $P < 0.001$ ), compared with controls. Thompson and Zhang (1991) reported a reduction in ACF in PA fed rats. The anticarcinogenic effect of PA is greater when PA is administered to the animals via drinking water as compared to PA in feed as shown by Challa et al. (1997). PA could form insoluble complexes with proteins and other macromolecules in the diet and thus render them less available (Thompson, 1993; Harlan and Oberleas, 1987). Hirose et al. (1991), did not

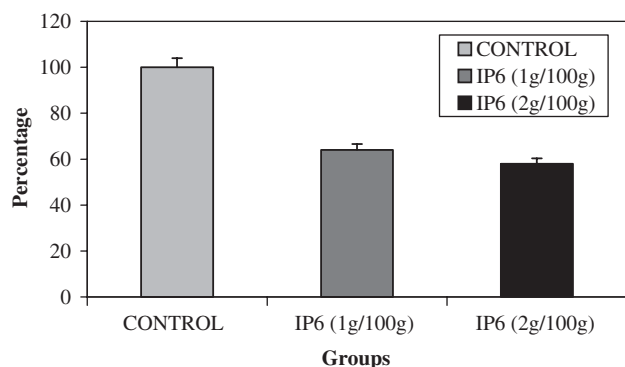


Fig. 1. Incidence (g/100 g) of azoxymethane-induced aberrant crypt foci (ACF) in colon of Fisher 344 male rats. Values are means  $\pm$  SEM,  $n = 15$ /group. Groups—Control, IP<sub>6</sub> (1 g/100 ml), IP<sub>6</sub> (2 g/100 ml).

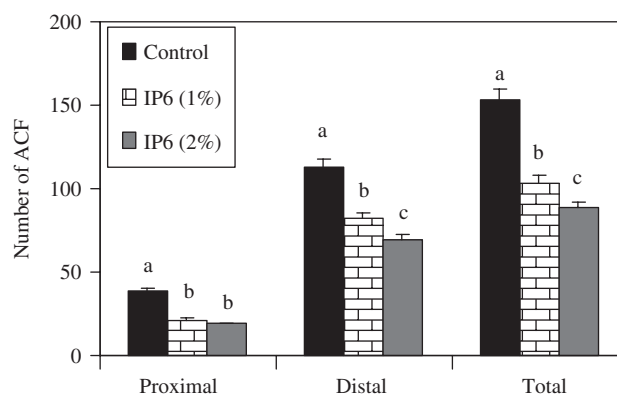


Fig. 2. Effect of 1 and 2 g/100 g phytic acid on azoxymethane-induced colonic aberrant crypt foci (ACF) in Fisher 344 male rats. Values are means  $\pm$  SEM,  $n = 15$ /group. Groups—Control, IP<sub>6</sub> (1 g/100 ml), IP<sub>6</sub> (2 g/100 ml).

Table 3

Effect of 1 and 2 g/100 g phytic acid on number of total crypts in colon of rats for 13 week

Group	Proximal	Distal	Total
Control	128.6 $\pm$ 2.0 <sup>a</sup>	398.8 $\pm$ 6.6 <sup>a</sup>	529.4 $\pm$ 7.2 <sup>a</sup>
1 g/100 g IP <sub>6</sub>	65.8 $\pm$ 2.9 <sup>b</sup>	274.8 $\pm$ 8.3 <sup>b</sup>	341.3 $\pm$ 2.7 <sup>b</sup>
2 g/100 g IP <sub>6</sub>	64.9 $\pm$ 2.8 <sup>b</sup>	238.3 $\pm$ 5.2 <sup>c</sup>	303.2 $\pm$ 5.1 <sup>c</sup>

Values are means  $\pm$  SEM, means in a column without a common letter differ (Tukey's test),  $P < 0.05$ .

$n = 15$ /group.

observe a significant inhibition of colon tumors by dietary IP<sub>6</sub> which is contrary to the results observed in this study which could be due to the fact that they used tumors as the end point.

### 3.1.3. Cell culture

After 24 h of incubation, LDH release (relative to control) was dose dependent and ranged from 13.55% to 44.70% (Fig. 3) in cells treated with 0.25–4.0 g mol/l of IP<sub>6</sub>. At concentrations of  $> 5.0$  g mol/l gelling of cells (complete lysis) was seen. Similar observations were reported by Shamsuddin (1995) in MCF-7 and MDA-MB-231 human mag mol/Lary carcinoma cell lines. After 48 h of incubation complete cell death was seen when observed under a microscope. Histone-associated DNA fragmentation in IP<sub>6</sub>-treated cells was also dose dependent with absorbance values ranging from 0.293 to 1.23 (Fig. 4). CaCo-2 cells grown on slide flasks showed membrane blebbing (zeiosis) characteristic of apoptotic activity (Fig. 5).

An increase in LDH release, histone-associated DNA fragmentation and cell membrane blebbing in CaCo-2 cells treated with PA (IP<sub>6</sub>) may indicate that one of the mechanisms by which IP<sub>6</sub> exerts its antitumorigenic effect which is observed in animal models is by inducing apoptotic activity in the tumor cell. Another mechanism may be by boosting the activity of natural killer (NK) cells which are immune system cells that can kill tumor cells



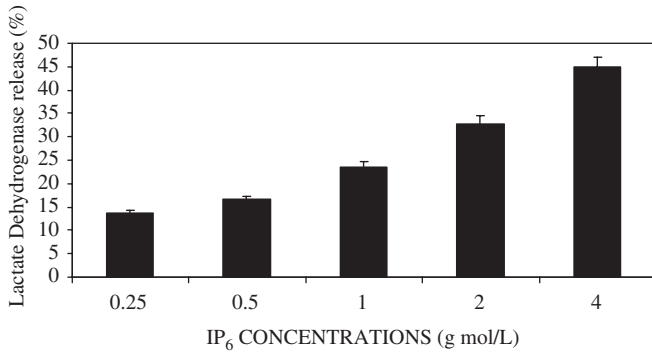


Fig. 3. Lactate dehydrogenase release (LDH) caused by IP<sub>6</sub> in CaCo-2 cells.

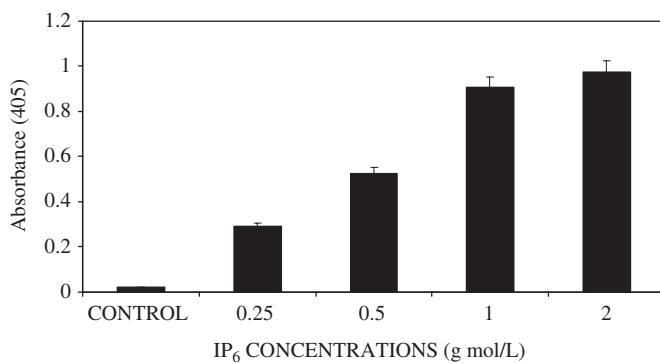


Fig. 4. Histone-associated DNA fragmentation caused by IP<sub>6</sub> in CaCo-2 cells.

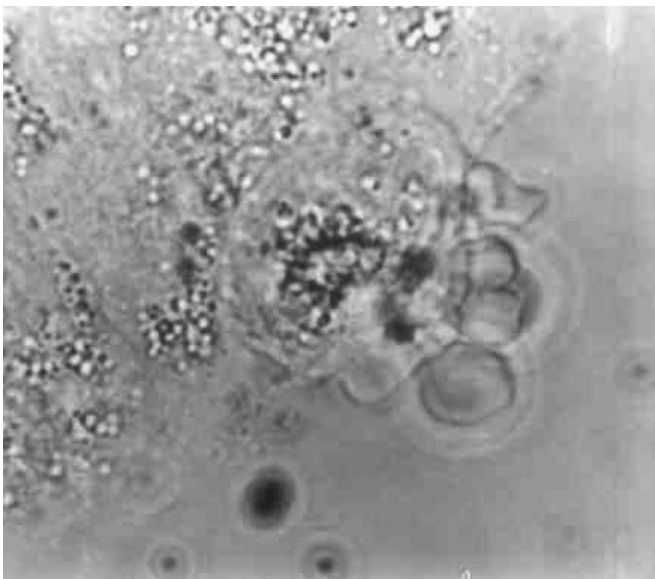


Fig. 5. Membrane blebbing (Zieosis) characteristic of apoptotic activity.

(Shamsuddin, 1992). Huang et al. (1997) demonstrated the mechanism of IP<sub>6</sub> at the biochemical level with its involvement in a system of messenger cells or the signal

transduction pathway which can be activated by tumor promoters. The tumor promoters activate protein 1 (AP-1), a crucial component of tumor growth, by way of an enzyme: phosphatidyl inositol-3 (PI-3) kinase. IP<sub>6</sub> is found to have a structure very similar to a potent inhibitor of the enzyme PI-3 kinase, thus working against, or inhibiting the tumor by blocking the action of the enzyme. IP<sub>6</sub> could also reduce the risk of cancer by increasing the activity of glutathione S-transferase which is a key enzyme responsible for detoxifying various chemicals including carcinogens (Challa et al., 1997).

Some possible mechanisms of the antineoplastic activity of PA are that PA (IP<sub>6</sub>) functions as an antioxidant and this function occurs by chelating iron by occupying all available iron coordinating sites, thus inhibiting OH generation from the Fenton reaction and the subsequent lipid peroxidation and DNA damage. IP<sub>6</sub> could reduce the active oxygen species-mediated carcinogenesis and cell injury via its antioxidative function (Shamsuddin, 1995) as it is reported to have the singular ability to remove O<sub>2</sub> without generating oxy-radicals. They also reported that dietary antioxidants elevated glutathione-S-transferase levels in animals treated with carcinogens, and that elevation of the Phase II enzymes activity could be another route of the anticarcinogenic effect of PA.

IP<sub>6</sub> was shown to increase the expression of the tumor-suppressor gene p53 by up to 17 fold (Saied & Shamsuddin, 1997). Along with this increased p53 expression, there was a reduction in cell proliferation and enhanced cell maturation or differentiation. Since the decreased expression of p53 is also associated with the resistance of tumors to chemotherapy, IP<sub>6</sub> could be used as an adjuvant to enhance p53 expression with other chemotherapeutic drugs.

#### 4. Conclusions

The results of this experiment clearly showed that oral administration of phytic acid (PA) (1 and 2 g/100 ml) in water significantly ( $P < 0.05$ ) reduced AOM-induced aberrant crypt foci in Fisher 344 male rats. Doubling the dose from 1 to 2 g/100 ml did not double the effect. This could be due to other physiological factors influencing the outcome which may result in an increased reduction rather than an exact doubling of the reduction of ACF incidence. The number of ACF were significantly higher in the distal colon compared with the proximal colon. These data are consistent with the reports that the distal colon shows a greater incidence of colorectal cancer than the proximal colon in humans. The group fed PA at 2 g/100 ml had a significantly greater reduction in ACF compared to the group fed 1 g/100 ml. An increase in LDH release, histone-associated DNA fragmentation and cell membrane blebbing in Caco-2 cells treated with PA (IP<sub>6</sub>) was also seen which indicates that IP<sub>6</sub> might exert anticarcinogenic activity through induction of apoptosis.

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